

Involvement of MAPKs in ICAM-1 Expression in Glomerular Endothelial Cells in Diabetic Nephropathy

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Inflammatory processes are involved in the pathogenesis of diabetic nephropathy. The aim of this study was to clarify the role of mitogen-activated protein kinase (MAPK) pathways for induction of intercellular adhesion molecule-1 (ICAM-1) expression in glomerular endothelial cells under diabetic conditions. We examined the expression of ICAM-1 in the kidneys of experimental diabetic rats. Human glomerular endothelial cells (GE cells) were exposed to normal glucose concentration, high glucose concentration (HG), or high mannitol concentration (HM), and then the expression of the ICAM-1 protein and the phosphorylation of the 3 subfamilies of mitogen-activated protein kinase (MAPK) were determined using Western blot analysis. Next, to evaluate the involvement of MAPKs in HG- or HM-induced ICAM-1 expression, we preincubated GE cells with the inhibitors for ERK, p38 or JNK 1h prior to the application of glucose or mannitol. Expression of ICAM-1 was increased in the glomeruli of diabetic rats. Both HG and HM induced ICAM-1 expression and phosphorylation of ERK1/2, p38 and JNK in GE cells. Expression of ICAM-1 was significantly attenuated by inhibitors of ERK, p38 and JNK. We conclude that activation of ERK1/2, p38 and JNK cascades may be involved in ICAM-1 expression in glomerular endothelial cells under diabetic conditions.

Key words: diabetic nephropathy, ICAM-1, ERK, p38 MAPK, JNK

Diabetic nephropathy is a major cause of end-stage renal failure worldwide. Several mechanisms have been postulated for the development of diabetic nephropathy. Glomerular hyperfiltration, which is noted in the early stage of diabetic nephropathy, causes glomerular injury in type 1 and type 2 diabetes [1]. Hyperglycemia induces accumulation of advanced glycation end products (AGEs) [2], acceleration of the polyol pathway [3], activation of pro-

tein kinase C [4], an increases of oxidative stress [5], and overexpression of transforming growth factor (TGF)- β [6]. In addition to these factors, recent studies have shown the critical roles of inflammatory processes in the development of diabetic nephropathy.

Infiltration of macrophages into the glomeruli and interstitium is one of the characteristic features of diabetic nephropathy in addition to mesangial matrix expansion and interstitial fibrosis. Furuta *et al.* [7] reported that the number of macrophages in the glomeruli was significantly higher in moderate-stage than advanced-stage diabetic glomerulosclerosis. It has also been demonstrated that glomerular and interstitial

injury is associated with macrophage infiltration in both type 1 and type 2 diabetes [8, 9]. These results suggest the pathological role of macrophages in the development of diabetic nephropathy.

Intercellular adhesion molecule-1 (ICAM-1) is one of the major molecules that promotes leukocyte attachment to the vascular endothelium and their transmigration by its binding to β_2 -integrins on leukocyte cell surfaces. We have reported in a series of studies that ICAM-1 is upregulated and mediates macrophage infiltration in diabetic kidney tissues [10–12]. We have also demonstrated that ICAM-1-deficient mice are protected from renal injury after induction of diabetes [13]. Furthermore, we have recently shown that anti-inflammatory agents could ameliorate experimental diabetic nephropathy through inhibition of ICAM-1 expression and macrophage infiltration [14–17].

It has been reported that exposure of the vascular endothelium to elevated glucose concentrations induces expression of ICAM-1 *in vitro*. A recent study has also shown that short-term (24-h) exposure of human umbilical vein endothelial cells (HUVEC) to high glucose concentrations resulted in the enhanced expression of ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and endothelial-leukocyte adhesion molecule-1 (ELAM-1) [18]. Another study demonstrated that high glucose can upregulate ICAM-1 protein and mRNA expression in rat mesangial cells, in part through osmotic effects, and that this upregulation of promotes leukocyte adhesion [19].

Mitogen-activated protein kinase (MAPK) cascades comprise one of the major signaling systems by which cells transduce and integrate diverse intracellular signals. The three subfamilies of MAPKs are the extracellular signal-regulated kinases (ERKs), the p38 MAPKs, and the c-Jun NH₂-terminal kinases (JNK). ERKs are activated primarily in response to proliferated stimuli, whereas the other MAPKs are activated primarily in response to inflammatory and stressful stimuli, including oxidant and osmotic stresses.

It has been well established that high glucose induces expression of ICAM-1 in different cell types, but little is known about the intracellular signaling pathways leading to adhesion molecule expression. In this study, we aimed to clarify the involvement of the 3 subfamilies of MAPKs in the process of ICAM-1 expression in glomerular endothelial cells under dia-

betic condition. We evaluated the role of the MAPKs in the expression of ICAM-1 induced by high glucose or high mannitol in cultured glomerular endothelial cells.

Materials and Methods

Reagents and antibodies. Chemicals and reagents including D-glucose and D-mannitol were obtained from Sigma Chemical (St. Louis, MO, USA), unless otherwise noted. Cell culture medium (EBM-2) and growth supplements were purchased from Clonetics (Walkersville, MD, USA). Fetal bovine serum (FBS) was obtained from CanSera International (ON, Canada). Mouse monoclonal anti-rat ICAM-1 antibody (1A29) was purchased from Seikagaku Kogyo (Tokyo, Japan). FITC-conjugated goat anti-mouse IgG was obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Mouse monoclonal anti-ICAM-1 antibody was purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Rabbit polyclonal anti-phospho-p38 antibody, anti-p38 antibody and anti-GAPDH antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal phospho-MAP kinase 1/2 (Erk1/2) antibody, anti-MAP kinase 1/2 (Erk1/2) antibody, phospho-JNK1 antibody and anti-JNK/SAPK were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Alexa Fluor 488 anti-mouse IgG antibody was obtained from Molecular Probes (Eugene, OR, USA). ERK inhibitor II, p38 inhibitor (SB203580) and JNK inhibitor (SP600125) were purchased from Calbiochem (Darmstadt, Germany). Horseradish peroxidase (HRP)-linked anti-mouse and anti-rabbit IgG antibodies and the HRP Western blot detection kit were obtained from GE Healthcare Biosciences (Piscataway, NJ, USA).

Experimental protocol. Male Sprague-Dawley rats aged 5 wk were divided into 2 groups: nondiabetic control rats (non-DM, n=5) and streptozotocin (STZ)-induced diabetic rats (DM, n=5). DM rats were injected intravenously with STZ (65mg/kg body wt) in citrate buffer (pH 4.5). Blood glucose levels were determined at 3 and 7 days after STZ injection, and only rats with blood glucose concentrations > 300mg/dl were used in the study. Non-DM rats received an injection of citrate buffer alone. Rats of

the non-DM and DM groups received normal chow. All rats had free access to standard chow and tap water. All procedures were performed according to the Guidelines for Animal Experiments at Okayama University Medical School, the Japanese Government Animal Protection and Management Law (No. 105), and the Japanese Government Notification on Feeding and Safekeeping of Animals (no. 6). All rats were killed 8 weeks after the induction of diabetes, and the kidneys were harvested. The tissues were embedded in optimal-cutting-temperature compound (Sakura Finetechnical, Tokyo, Japan) and immediately frozen in acetone cooled on dry ice.

Immunofluorescence staining for ICAM-1 in rat kidney. The expression of ICAM-1 was detected by indirect immunofluorescence as described previously [20, 21]. Briefly, fresh-frozen sections (4- μ m thick) were stained with a mouse monoclonal anti-rat ICAM-1 antibody (1A29) for 1 h at room temperature, and then stained with FITC-conjugated goat anti-mouse IgG for 30 min at room temperature. The stained sections were observed by a confocal laser fluorescence microscope (LSM-510; Carl Zeiss, Jena, Germany). The immunofluorescence intensities of glomerular ICAM-1 were quantified as follows: color images were obtained as TIF files by LSM-510. The brightness of each image file was uniformly enhanced and analyzed using Lumina Vision software (Mitani, Fukui, Japan). Image files (TIF) were inverted and opened in gray-scale mode.

Cell culture. Human glomerular endothelial cells (GE cells) were obtained from Applied Cell Biology Research Institute (Kirkland, WA, USA) and cultured in EBM-2 complete medium containing 5.5 mM D-glucose and 10% FBS in a 5%-CO₂ incubator at 37°C. Cells from each primary flask (passage 4 to 8) were detached with trypsin/EDTA, re-suspended in fresh culture medium, and passaged into gelatinized 6-well plates for Western blot analysis or 12-well plates with gelatinized coverslips for immunofluorescence analysis. GE cells were grown to subconfluence (80%) and then rendered quiescent in EBM-2 containing 0.1% FBS. After 24h, GE cells were exposed to the following experimental conditions: 1) 5.5mM D-glucose (normal glucose; NG), 2) 30mM D-glucose (high glucose; HG), or 3) 5.5mM D-glucose plus 24.5mM D-mannitol (high mannitol, HM), for 10, 30 and 60 min and 12 and 24h. When inhibitors were

used, cells were preincubated with 1 μ M ERK inhibitor II or 0.5 μ M SB203580 or 0.1 μ M SP600125 one hour prior to the application of D-glucose or D-mannitol.

Immunofluorescence microscopy. Cells on coverslips were fixed in 3.7% paraformaldehyde in PBS for 20 min, washed three times with PBS, and blocked with 5% bovine serum albumin (BSA) in tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 30 min. Incubations with mouse monoclonal anti-ICAM-1 antibody were performed in blocking solution (5% BSA in TBS-T) for 1 h at room temperature. After three washes with TBS-T, cells were incubated with appropriate secondary antibodies conjugated to immunofluorescent dyes in blocking solution for 1 h at room temperature. After three washes with PBS, the coverslips were mounted using Pristine Mount (Pharma, Tokyo). The sections were observed under a BIOZERO fluorescent microscope BZ-8000 (Keyence, Osaka, Japan).

Western blot analysis. Western blot analysis was performed as described [20, 21]. Briefly, after washing with PBS, cells prepared on 6-well plates as described in the section of cell culture were lysed with 300 μ l per well of cell lysis buffer containing 10 mM Tris (pH 7.4), 1% Triton X-100, 0.5% Nonidet P-40, 150mM NaCl, 1mM EDTA, 0.2mM EGTA, 0.2mM vanadate, 0.2mM PMSF, and 0.5% phosphatase inhibitor cocktail. The protein concentration of the supernatant was measured with an RC DC protein assay kit (Bio-Rad, Hercules, CA, USA). Total cell lysates were cleared by centrifugation and boiled with the same amount of 3 \times SDS sample buffer for 5 min, then subjected to 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to PVDF membranes by electrotransfer. The blots were subsequently blocked with 5% BSA in TBS-T at room temperature for 1 h, and then incubated at 4°C overnight with the proper primary antibody at the dilutions recommended by the supplier. After washing 3 times for 10 min with TBS-T, the membrane was incubated with a 1: 1000 dilution of HRP-linked anti-mouse or rabbit IgG secondary antibody at room temperature for 1 h. The blots were then visualized with the ECL Western blot detection system. If membranes needed to be reprobed, they were incubated in reprobing buffer containing 62.5mM Tris (pH 6.8), 2% deoxy-

cholate, and 100mM mercaptoethanol at 70°C for 30min. After four 10-min washes with TBS-T, membranes were incubated with another primary antibody and then visualized again. The amount of detected proteins was analyzed using Image Quant (Molecular Dynamics, Chicago, IL, USA) software.

Statistical analysis. Results are expressed as means \pm SD of 3 to 6 independent experiments. Stimulated samples were compared with controls by unpaired Student's *t* test. For multiple-group comparisons, one-way analysis of variance (ANOVA) followed by the post hoc Fisher's test was performed using StatView software. $P < 0.05$ was considered statistically significant.

Results

Expression of ICAM-1 in kidneys. HbA1c and urinary albumin excretion (UAE) are significantly higher in diabetic rats compared with nondiabetic rats (HbA1c; 13.6% vs 3.6%, UAE; 2090 μ g/day vs 65.4 μ g/day, $p < 0.05$). In diabetic rats, ICAM-1 expression was increased in glomeruli compared with nondiabetic rats (Fig. 1).

Effect of high glucose and high mannitol on ICAM-1 expression in GE cells. On immunofluorescence microscopy, ICAM-1 was seen in a fine granular pattern on the surface of GE cells. Although

ICAM-1 was expressed at low levels in the basal state (NG), its expression was significantly increased in HG and HM (Fig. 2). Western blot analysis also revealed that exposure to HG and HM induced ICAM-1 protein expression in a time-dependent manner in GE cells (Fig. 3). Exposure to HG increased ICAM-1 protein expression 4.18 ± 0.84 -fold after 12h and 5.83 ± 1.22 -fold after 24h compared with that at baseline (Fig. 3A). Similarly, exposure to HM increased ICAM-1 protein 2.39 ± 0.32 -fold after 12h and 5.36 ± 1.51 -fold after 24h (Fig. 3B).

Effect of HG and HM on phosphorylation of ERK1/2 in GE cells. To examine whether ERK1/2 phosphorylation is mediated by HG and HM in GE cells, the activation of these kinases was assayed by Western blot using an antibody specific for the phosphorylated, active forms of ERK1/2. Both HG and HM stimulated phosphorylation of ERK1/2 in GE cells (Fig. 4). The maximal response was obtained within 10min and then declined.

Effect of HG and HM on phosphorylation of p38 in GE cells. To determine whether p38 phosphorylation is mediated by HG and HM in GE cells, activation of this kinase was assayed by Western blot using an antibody specific for the phosphorylated, active forms of p38. Both HG and HM stimulated phosphorylation of p38 in GE cells (Fig.

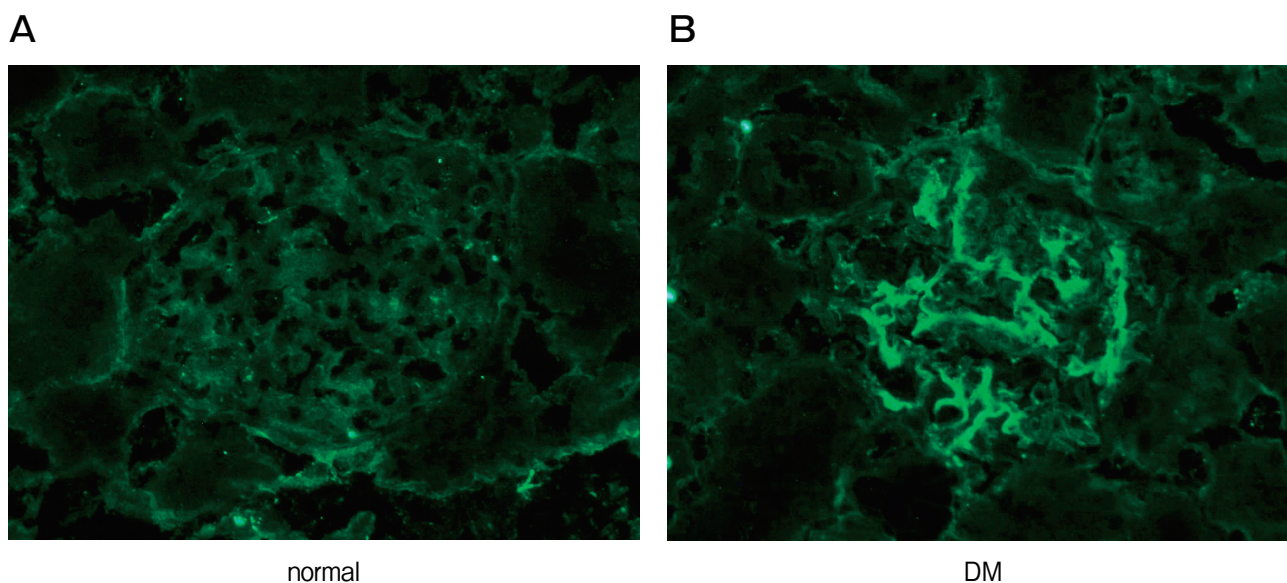


Fig. 1 Immunofluorescence staining for ICAM-1 in the glomeruli of nondiabetic (A) and diabetic rats (B).

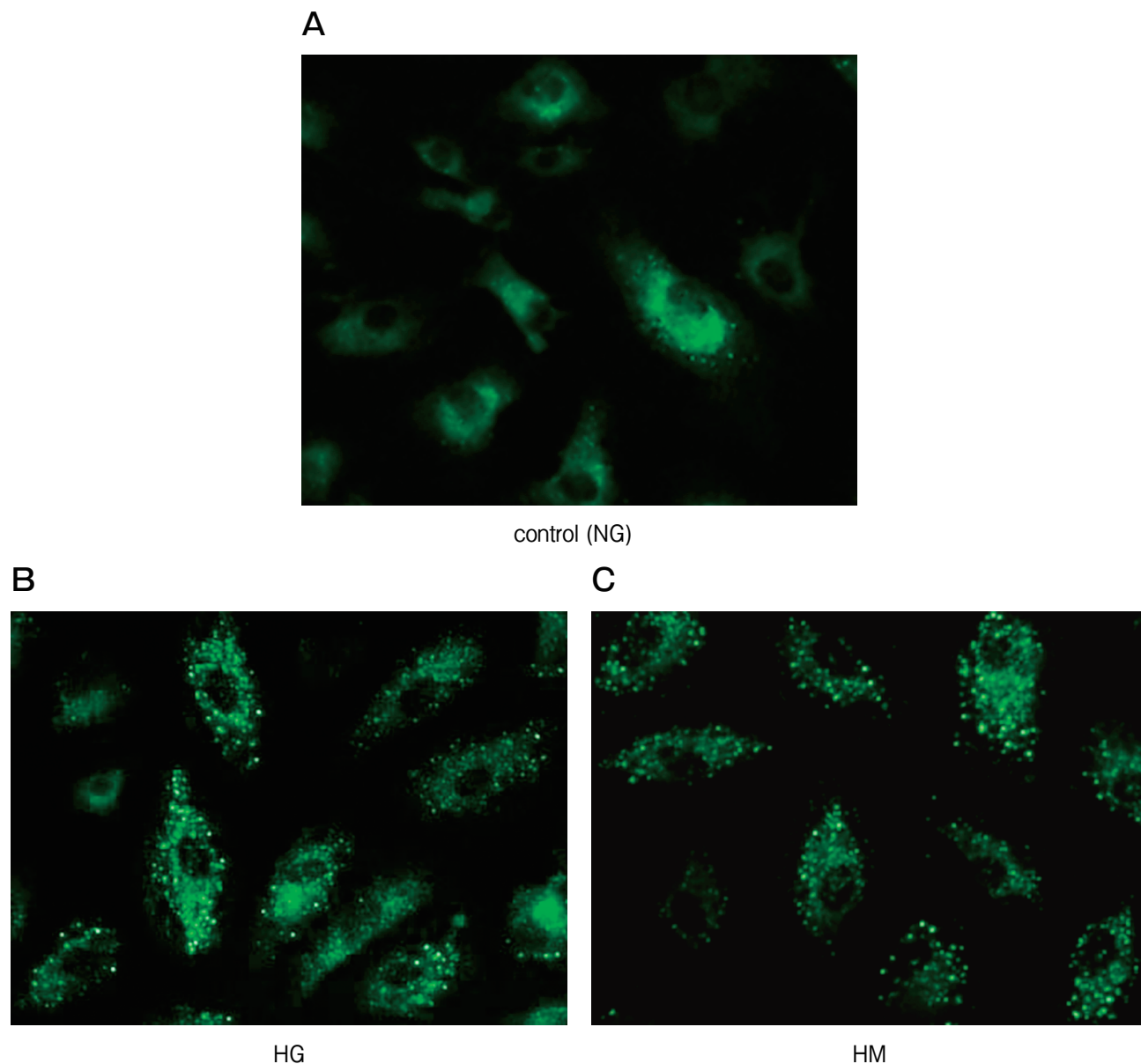


Fig. 2 Immunofluorescence staining for ICAM-1 in GE cells. GE cells were incubated with 5.5 mM D-glucose (**A**), 30 mM D-glucose (**B**) and 5.5 mM D-glucose plus 24.5 mM D-mannitol (**C**) for 24 h.

5). The maximal response was achieved within 10 min and then slightly declined.

Effect of HG and HM on phosphorylation of JNK1 in GE cells. To examine whether JNK1 phosphorylation is mediated by HG and HM in GE cells, activation of this kinase was assayed by Western blot using an antibody specific for the phosphorylated, active forms of JNK1. Both HG and HM

stimulated phosphorylation of JNK1 in GE cells (Fig. 6), and the maximal response was obtained within 10 min and then declined.

Involvement of ERK, p38 and JNK for HG- and HM-induced expression of ICAM-1 in GE cells. To determine whether ERK, p38 and JNK are involved in HG-induced ICAM-1 expression in GE cells, ERK inhibitor II, p38 inhibitor (SB203580)

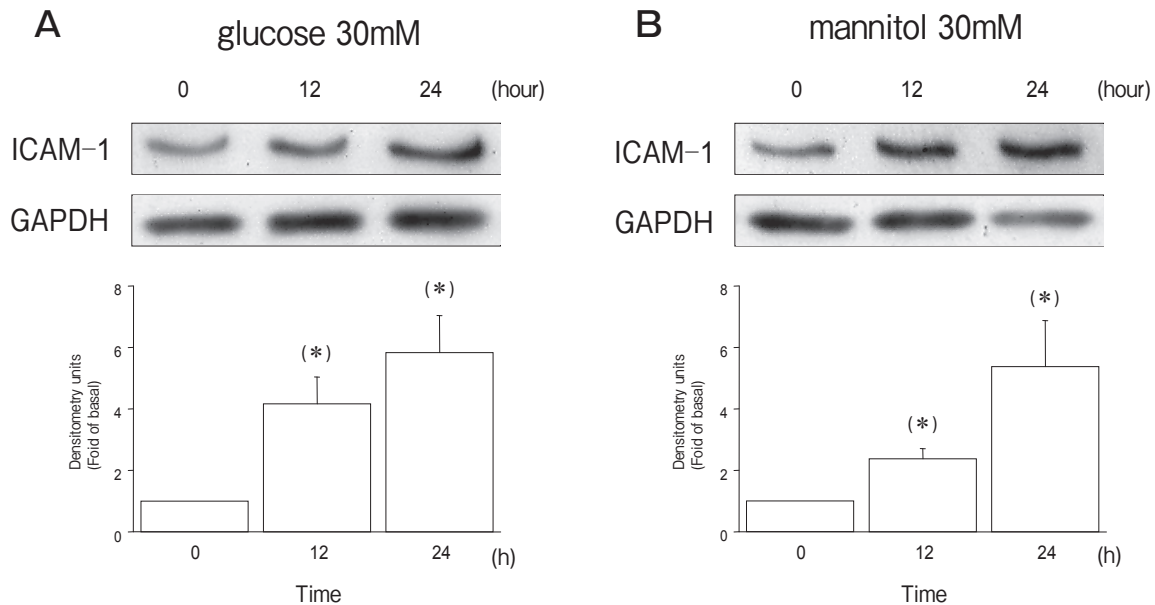


Fig. 3 The increase of ICAM-1 expression in GE cells induced by high glucose and high mannitol. GE cells were incubated with 30 mM D-glucose (HG) (A) and 5.5mM D-glucose plus 24.5mM D-mannitol (HM) (B) for 12 and 24h. Total cell lysates were analyzed by immunoblotting with anti-ICAM-1 antibody, as indicated. The histogram represents the means \pm SE of densitometric scans of ICAM-1 protein bands from 5 independent experiments, normalized by comparison with GAPDH and expressed as ratios of the basal level (0h). * $p < 0.05$.

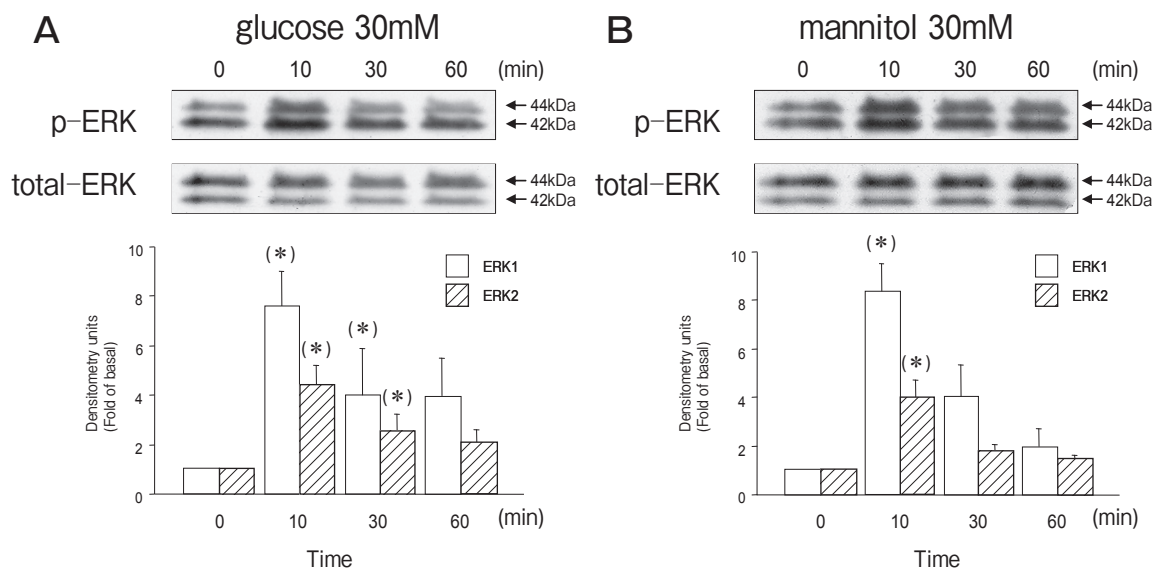


Fig. 4 The effect of HG and HM on phosphorylation of ERK1/2 in GE cells. **A**, GE cells were incubated with 30mM D-glucose for the indicated time periods. The resultant total cell lysates were probed with anti-phospho-ERK1/2 antibody and subsequently reprobed with anti-ERK1/2 antibody. The histogram represents the means \pm SE of densitometric scans of phospho-ERK1/2 bands from 3 independent experiments, normalized by comparison with total ERK1/2 bands and expressed as ratios of the basal level (0min). * $p < 0.05$. **B** In the same way, total cell lysates prepared from HM-stimulated GE cells were analyzed by immunoblotting.

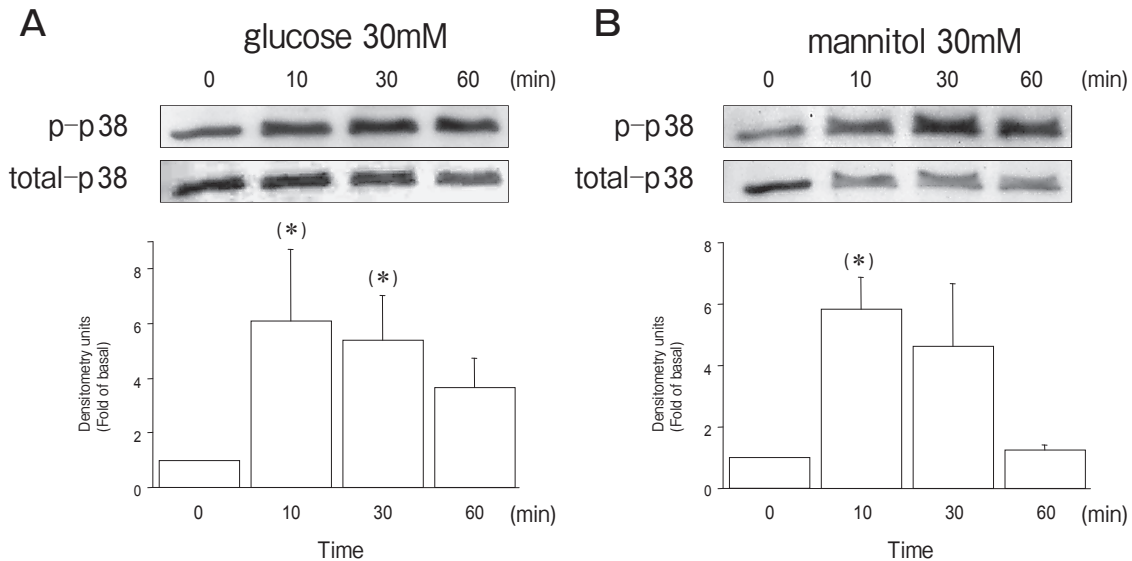


Fig. 5 The effect of HG and HM on phosphorylation of p38 in GE cells. **A**, GE cells were incubated with 30mM D-glucose for the indicated time periods. The resultant total cell lysates were probed with anti-phospho-p38 antibody and subsequently reprobated with anti-p38 antibody. The histogram represents the means \pm SE of densitometric scans of phospho-p38 bands from 3 independent experiments, normalized by comparison with the total p38 band and expressed as ratios of the basal level (0min). * $p < 0.05$. **B**, In the same way, total cell lysates prepared from HM-stimulated GE cells were analyzed by immunoblotting.

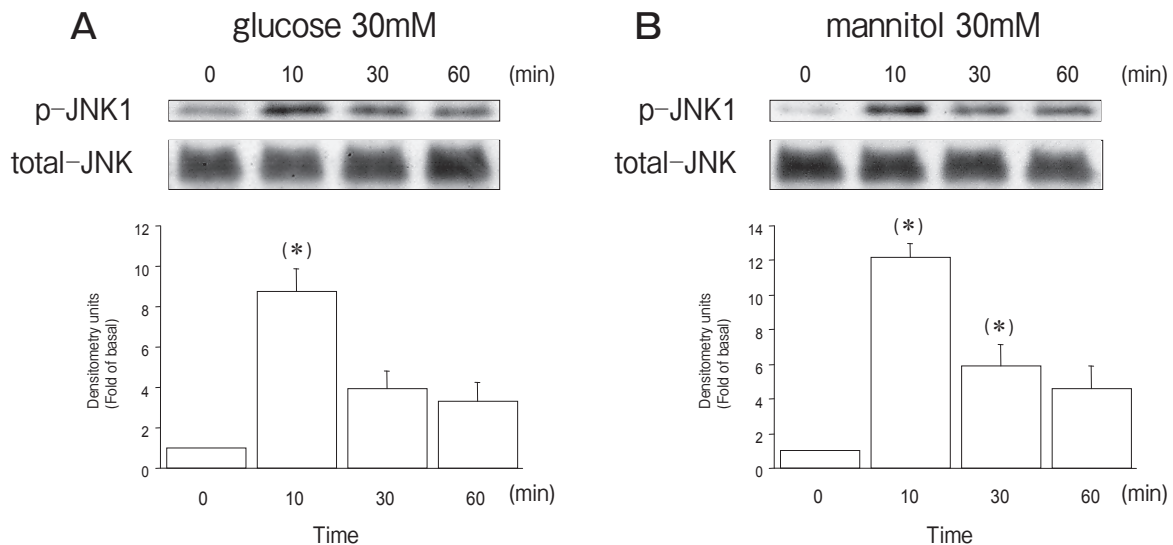


Fig. 6 The effect of HG and HM on phosphorylation of JNK1 in GE cells. **A**, GE cells were incubated with 30mM D-glucose for the indicated time periods. The resultant total cell lysates were probed with anti-phospho-JNK1 antibody and subsequently reprobated with anti-JNK antibody. The histogram represents the means \pm SE of densitometric scans of phospho-JNK1 bands from 3 independent experiments, normalized by comparison with total JNK bands and expressed as ratios of the basal level (0min). * $p < 0.05$. **B**, In the same way, total cell lysates prepared from HM-stimulated GE cells were analyzed by immunoblotting.

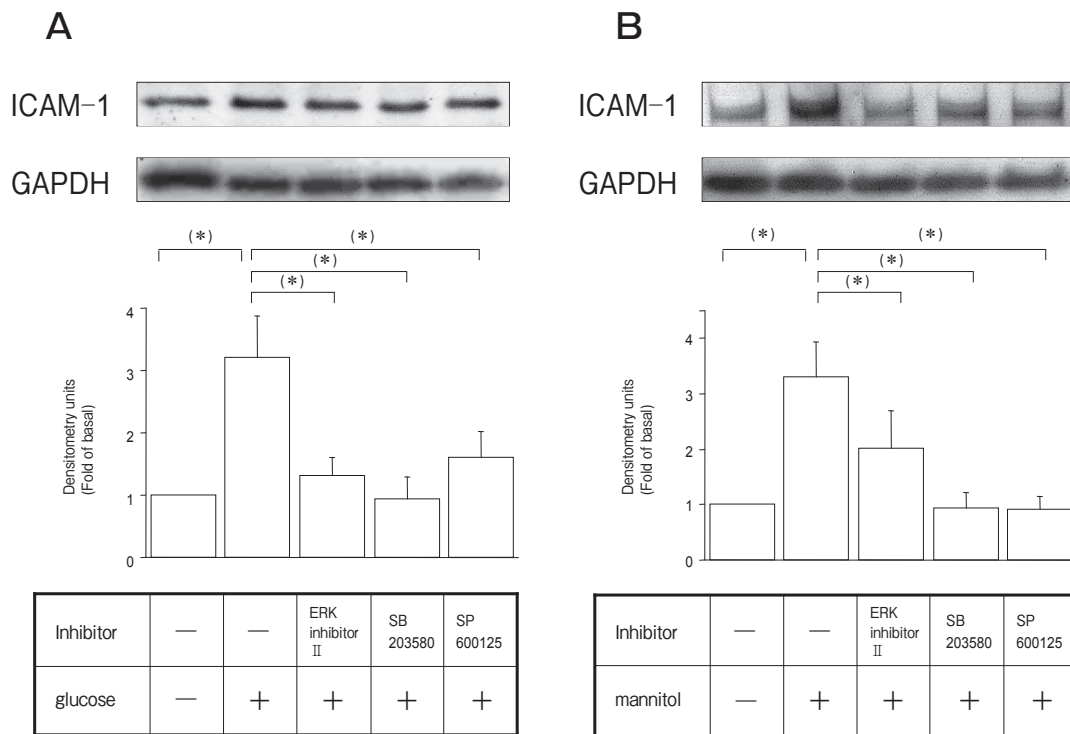


Fig. 7 Involvement of ERK, p38 and JNK for HG- and HM-induced expression of ICAM-1 in GE cells. **A**, Cells were preincubated with ERK inhibitor II, SB203580 and SP600125 for 1h, and then incubated with HG for 24h. Total cell lysates were analyzed by immunoblotting with anti-ICAM-1 antibody, as indicated. The histogram represents the means \pm SE of densitometric scans of ICAM-1 protein bands from 6 independent experiments, normalized by comparison with the GAPDH band and expressed as ratios of the basal level. * p < 0.05. **B**, In the same way, total cell lysates prepared from HM-stimulated GE cells were analyzed by immunoblotting.

and JNK inhibitor (SP600125) were used. As shown in Fig. 7A, pretreatment with these inhibitors for 1h prior to exposure to HG for 24h caused an attenuation of ICAM-1 expression. The relative amount of ICAM-1 was as follows: NG, 1.00; HG, 2.96 ± 0.46 ; HG+ERK inhibitor II, 1.09 ± 0.24 ; HG+SB203580, 0.76 ± 0.26 ; HG+SP600125, 1.73 ± 0.28 . Similarly, we evaluated the effects of ERK inhibitor II, SB203580, and SP600125 on HM-induced ICAM-1 expression. As shown in Fig. 7B, each treatment inhibited the increase of ICAM-1 expression, but ERK inhibitor II showed weak inhibition compared to the other inhibitors. The relative amount of ICAM-1 was as follows: NG, 1.00; HM, 3.31 ± 0.62 ; HM+ERK inhibitor II, 2.02 ± 0.69 ; HM+SB203580, 0.95 ± 0.28 ; HM+SP600125, 0.91 ± 0.23 .

Discussion

In this study, we showed that exposure to HG and HM induced ICAM-1 protein expression in a time-dependent manner in GE cells. In addition, we found that both HG and HM stimulated phosphorylation of ERK1/2, p38 and JNK1. HG-induced ICAM-1 expression was significantly attenuated by ERK inhibitor II, SB203580 (an inhibitor of p38), and SP600125 (an inhibitor of JNK). HM-induced ICAM-1 expression was also attenuated by these inhibitors. These results demonstrate that the activation of the ERK1/2, p38 and JNK cascades, at least in part, is essential for HG- and HM-induced ICAM-1 expression.

The expression of adhesion molecules including ICAM-1 constitutes the basis for leukocyte migration into endothelial cells processing endothelial inflamma-

tion. In diabetic patients, the induction of adhesion molecules on the surface of endothelial cells is one of the first steps in HG-mediated endothelial dysfunction. Previous studies have suggested several possible mechanisms of ICAM-1 induction in diabetic renal tissues: 1) ICAM-1 is induced by inflammatory cytokines such as tumor necrosis factor- α , interleukin-1, and interferon- γ [22]; 2) Activation of protein kinase C (PKC) results in upregulation of ICAM-1 in endothelial cells [23]; 3) AGEs enhance the expression of cell adhesion molecules [24, 25]; 4) Shear stress stimulates the induction of ICAM-1 [26]; 5) Oxidative stress increases ICAM-1 expression in glomeruli [27]; and 6) Osmotic agents upregulate ICAM-1 expression in rat mesangial cells [19] and in HUVEC [28]. In this study, we also showed that increased ICAM-1 expression could be induced by HG and high concentrations of another osmotic agent, mannitol. Therefore, in GE cells, ICAM-1 expression can be upregulated by hyperglycemia, at least in part by an osmotic effect.

The effect of glucose on the activation of MAPKs has been examined in different cell types. Previous studies demonstrated that the exposure to glucose activated both ERKs and p38, but JNK was not phosphorylated in rat aortic smooth muscle cells [29], rat mesangial cells [30] and bovine aortic endothelial cells [31]. On the other hand, one study reported that neither ERKs nor p38 activity was increased by glucose in bovine pulmonary artery endothelial cells [32], and another study showed that HG was capable of inducing JNK but not ERKs and p38 in HUVECs [33]. Furthermore, a recent study demonstrated that not HG but glucose-induced cytokines activate MAPKs in human retinal endothelial cells [34]. These data suggest that results vary in different cell types. In the present study, we showed that ERK1/2, p38 and JNK1 were all phosphorylated by HG in GE cells. This results are consistent with recent reports showing activation of MAPKs in human diabetic nephropathy [35–37]. Some of the effects of HG are attributed to an increase in osmolarity. Hyperosmolarity-induced activation of ERKs, JNK, and p38 has been also observed in different cell types, such as mouse inner medullary collecting duct cells [38], bovine aortic endothelial cells [39], and 3T3-L1 adipocytes [40]. It seems to be generally the case that p38 and JNK are activated by hyperosmotic stress, whereas

the response of ERKs to hyperosmotic stress varies with the cell type. In addition, different osmotic agents may induce MAPK activation differently. One study showed that neither ERKs nor p38 activity was increased by mannitol in HUVECs [41]. Another study demonstrated that mannitol was able to induce activation of ERKs, but no activation of p38 could be observed in GP8 rat brain endothelial cells [42]. Our study showed that ERK1/2, p38 and JNK1 were all phosphorylated by HM. Therefore, in GE cells, it seems that HM is one of the mediators of the activation of MAPKs observed in HG.

Our major finding was that all 3 subfamilies of MAPKs were involved in ICAM-1 expression induced by HG in GE cells. Several studies have demonstrated that ICAM-1 expression is induced by proinflammatory cytokines such as TNF- α and IL-1 β , either entirely or partly through MAPK signaling pathways [43, 44]. However, there are few reports about the involvement between MAPKs and ICAM-1 expression induced by high glucose itself. A recent study showed that HG-induced ICAM-1 expression was inhibited by selenium in HUVECs, which is partially mediated through the modulation of the p38 pathway [45]. Another recent study demonstrated that chronic p38 inhibition reduced ICAM-1 and VCAM-1 expression in the quadriceps muscle in diabetic rats [46]. We evaluated similar results regarding the involvement between p38 and HG-induced ICAM-1 expression in GE cells. On the other hand, there have been no previous reports evaluating involvement between HG-induced ICAM-1 expression and ERKs or JNK. To our knowledge, this is the first report to demonstrate not only p38 but also ERK- and JNK-mediated ICAM-1 expression induced by HG. This finding may support the protective role of MAPK inhibition in the reduction of the development of diabetic nephropathy via anti-inflammatory mechanisms.

In conclusion, our study suggests that ERKs, p38 and JNK signaling pathways are involved in the expression of ICAM-1 induced by both high glucose and high osmolarity in GE cells. All 3 subtypes of MAPKs may be involved in the activation of glomerular endothelial cells in a diabetic milieu.

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